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L4: Entry 1 of 7 File: USPT Mar 7, 2000

DOCUMENT-IDENTIFIER: US 6033861 A
TITLE: Methods for obtaining nucleic acid containing a mutation

DEPR:
Mutagenesis may be performed on whole organisms or on a selected tissue of an organism including but not limited to, for example, mutagenesis of germline cells of an organism, such as sperm stem cells or ova, mutagenesis of embryonic stem (ES) cells of an organism or introduction of a mutant gene into an organism which results in an increased frequency of mutations in the genome. Following mutagenesis of an organism, the organism may be analyzed directly for mutations, or it may be mated and the offspring analyzed for a mutation in a gene of interest. Obviously, it is preferred to analyze offspring in order to ensure that any mutation which is detected can be predictably passed on to further generations. Alternatively, following DNA analysis of a specific tissue for a mutation in a gene of interest, such as mutated ES clones in culture, the cells are transferred to the developing embryo. Mutagens and mutagenesis techniques which are applicable to organisms or cell mutagenesis are described below.

DEPR:
The zebrafish is a striped 2-inch long fish from the Ganges River. The zebrafish has been used as a genetic system and conditions for gamma-ray mutagenesis and screening are well-established (Chakrabarti et al., 1983, Brachydonio Genetics, 103: 109; Walker and Streisinger, 1983, Genetics, 103: 125). The advantages of zebrafish over the mouse for genetic analysis is its small size, the ability to house a large number of animals cheaply, and the large number of embryos produced from one female (usually a few hundred but as many as 1000 eggs). The time from fertilization to gastrulation is only about 5 hours at 28.degree. C.; somites form between 10-20 hours; and by 24 hours postfertilization, a recognizable animal with rudimentary eyes and brain has formed. Thus, the early development of this vertebrate takes only about as long as a phage plaque assay. Rossant et al.(1992, Genes Dev., 6: 1) describe mutational strategies for mutagenesis of zebrafish, including ENU mutagenesis.

DEPR:
Non-mammalian organisms, such as fish, nematodes, and insects, are particularly useful according to the invention in identifying mutations in genes which are suspected to play a role in early development of the organism, e.g., in embryonic

development, such as pattern-forming genes, limb-forming genes, or organ-forming genes.

DEPR:
UV light-induced mutations are largely single nucleotide alterations. However, because UV light does not penetrate an animal, it is used for inducing mutations in cells in culture, including embryonic stem (ES) cells, or on exposed tissues of an animal, e.g., eyes, skin.

DEPR:
McWhir et al. (1993, Nature Genetics, 5: 217) describe a mouse containing a defective DNA repair enzyme resulting from a mutation in the DNA repair gene ERCC-1. In nucleotide excision repair, damaged bases are removed with adjacent residues as an oligonucleotide and the resulting gap is then patched by repair synthesis. ERCC-1 is required for the incision step necessary to remove damaged DNA. Mice were generated containing the defective gene by targeting the excision repair cross complementing gene ERCC-1 in the embryonic stem cell line, HM-1. Homozygous ERCC-1 mutants died before weaning; however, heterozygous ERCC-1 mutants survived and were available for mating. It is contemplated according to the invention that a mammalian organism heterozygous for a mutant gene encoding a DNA repair enzyme may be used to screen for a mutation in a gene of interest.

DEPV:
procarbazine hydrochloride

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L4: Entry 2 of 7 File: USPT Jan 18, 2000

DOCUMENT-IDENTIFIER: US 6015670 A
TITLE: Methods for identifying a mutation in a gene of interest without a phenotypic guide using ES cells

BSPR:
As used herein, "organism" refers to a multicellular organism that undergoes development from an embryonic stage to an adult stage. The term "organism" may include insects, as well as vertebrates and invertebrates, the latter two categories of which fall within the term "animal". The invention is useful with respect to animals such as a nematode, a fish, such as a zebrafish, or a mammal, e.g., a rodent such as a mouse or a rat.

BSPR:
As used herein, the term "ES" cell refers to an embryonic stem cell.

DEPR:
The invention encompasses mutagenesis of whole organisms or of a selected tissue of an organism including but not limited to, for example, mutagenesis of germline cells of an organism, such as sperm stem cells or ova, or mutagenesis of embryonic stem (ES) cells of an organism, or introduction of a mutant gene into an organism which results in an increased frequency of mutations in the genome. Following mutagenesis of an organism, the organism may be analyzed directly for mutations, or it may be mated and the ofispring analyzed for a mutation in a gene of interest. Obviously, it is preferred to analyze offspring in order to ensure that any mutation which is detected can be predictably passed on to further generations. Alternatively, following DNA analysis of a specific tissue for a mutation in a gene of interest, such as mutated ES clones in culture, the cells are transferred to the developing embryo. Mutagens and mutagenesis techniques which are applicable to organisms or cell mutagenesis are described below.

DEPR:
The zebrafish is a striped 2-inch long fish from the Ganges River. The zebrafish has been used as a genetic system and conditions for gamma-ray mutagenesis and screening are well-established (Chakrabarti et al., 1983, Brachydonio Genetics 103:109; Walker and Streisinger, 1983, Genetics 103:125). The advantages of zebrafish over the mouse for genetic analysis is its small size, the ability to house a large number of animals cheaply, and the large number of

embryos produced from one female (usually a few hundred but as many as 1000 eggs). The time from fertilization to gastrulation is only about 5 hours at 28.degree. C.; somites form between 10-20 hours; and by 24 hours postfertilization, a recognizable animal with rudimentary eyes and brain has formed. Thus, the early development of this vertebrate takes only about as long as a phage plaque assay. Rossant et al., 1992, Genes Dev. 6:1, describe mutational strategies for mutagenesis of zebrafish, including ENU mutagenesis.

DEPR:
Non-mammalian organisms, such as fish, nematodes, and insects, are particularly useful according to the invention in identifying mutations in genes which are suspected to play a role in early development of the organism, e.g., in embryonic development, such as pattern-forming genes, limb-forming genes, or organ-forming genes.

DEPR:
McWhir et al., 1993, Nat. Genet. 5:217 describe a mouse containing a defective DNA repair enzyme resulting from a mutation in the DNA repair gene ERCC-1. In nucleotide excision repair, damaged bases are removed with adjacent residues as an oligonucleotide and the resulting gap is then patched by repair synthesis. ERCC-1 is required for the incision step necessary to remove damaged DNA. Mice were generated containing the defective gene by targeting the excision repair cross complementing gene ERCC-1 in the embryonic stem cell line, HM-1. Homozygous ERCC-1 mutants died before weaning; however, heterozygous ERCC-1 mutants survived and were available for mating. It is contemplated according to the invention that a mammalian organism heterozygous for a mutant gene encoding a DNA repair enzyme may be used to screen for a mutation in a gene of interest.

DEPR:
Sox-2 (both mouse Sox-2 and the human homolog (SOX2) is also a member of the Sox gene family. Sox-2 is involved in the transcriptional regulation of the FGF-4 gene. Fibroblast growth factor 4 (FGF-4) has been shown to be a signaling molecule whose expression is essential for postimplantation mouse development and, at later embryonic stages, for limb patterning and growth. The FGF-4 gene is expressed in the blastocyst inner cell mass and later in distinct embryonic tissues, but is transcriptionally silent in the adult. The mouse Sox2 nucleotide sequence is known (see Yuan et al., 199, Genes Dev. 9:2635) and has an open reading frame of 956 base pairs. The human SOX2 gene is expressed in fetal brain tissue. The SOX2 cDNA is 1085 bp long and contains an open reading frame of 317 amino acids, and displays a high degree of similarity with the mouse Sox2 gene. The nucleotide sequence of the SOX2 gene is known (Stevanovic et al., 1994, Mamm. Genome 5:640.) PCR primer pairs useful to generate amplimers in the Sox2 gene to detect gene mutations have the following sequences (F=forward; R=reverse):

DEPV:

procarbazine hydrochloride

ORPL:
Rossant et al., 1992, Of fin and fur: mutational analysis of vertebrate embryonic development, Genes Dev., 6: 1-13.

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L7: Entry 1 of 7 File: USPT Mar 7, 2000

DOCUMENT-IDENTIFIER: US 6033861 A
TITLE: Methods for obtaining nucleic acid containing a mutation

DEPR:
Many suitable methods for inducing mutations are known in the art. These include chemical mutagenesis, radiation, and retroviral or transposon insertion.

DEPR:
Mutagenesis may be performed on whole organisms or on a selected tissue of an organism including but not limited to, for example, mutagenesis of germline cells of an organism, such as sperm stem cells or ova, mutagenesis of embryonic stem (ES) cells of an organism or introduction of a mutant gene into an organism which results in an increased frequency of mutations in the genome. Following mutagenesis of an organism, the organism may be analyzed directly for mutations, or it may be mated and the offspring analyzed for a mutation in a gene of interest. Obviously, it is preferred to analyze offspring in order to ensure that any mutation which is detected can be predictably passed on to further generations. Alternatively, following DNA analysis of a specific tissue for a mutation in a gene of interest, such as mutated ES clones in culture, the cells are transferred to the developing embryo. Mutagens and mutagenesis techniques which are applicable to organisms or cell mutagenesis are described below.

DEPR:
Those mutagens or mutagenesis techniques which result in mutations which occur within a gene, i.e., a region of DNA from which RNA is transcribed, or within the regulatory elements controlling expression of the gene are most useful according to the invention. Chemical mutagens which result in such mutations include, but are not limited to, mutagens which are alkylating agents which cause single nucleotide changes.

DEPR:
Therefore, according to the invention, mutations are induced in an organism at a high enough frequency that the number of organisms needed to screen for a mutation in a gene of interest is not prohibitive. For example, it is useful according to the invention to induce mutations at a high frequency in order to decrease the number of organisms screened. ENU mutagenesis is particularly useful in the invention because, in the offspring of ENU mutagenized male mice, a mutation in any given gene will occur at a frequency of approximately 1 per 1000 mice. Thus,

approximately 1000 mice are screened in order to detect a mutation in a particular gene. Although the ratio of 1/1000 has been calculated in the prior art based on phenotypic assays, it is the only way of assessing the relative mutational frequencies of mutagens or mutagenesis techniques useful according to the invention, as direct DNA analysis of the frequencies of mutations induced by a given mutagen or mutagenesis technique has not been performed. Because phenotypic mutation frequencies are based on DNA mutations which alter or destroy the function of a protein such that it causes a phenotypic change, the number of changes in the DNA of these mice in a given gene will be higher than 1/1000 due to "silent" mutations, i.e., which do not result in a phenotypic change. The same type of mutation frequency is obtained using other chemical mutagens, such as MNU, PRC, and MMS. Additional mutagens which may be considered equally useful according to the invention include chlorambucil and melphalan, and those listed below and in Table 1.

DEPR:
The zebrafish is a striped 2-inch long fish from the Ganges River. The zebrafish has been used as a genetic system and conditions for gamma-ray mutagenesis and screening are well-established (Chakrabarti et al., 1983, Brachydonio Genetics, 103: 109; Walker and Streisinger, 1983, Genetics, 103: 125). The advantages of zebrafish over the mouse for genetic analysis is its small size, the ability to house a large number of animals cheaply, and the large number of embryos produced from one female (usually a few hundred but as many as 1000 eggs). The time from fertilization to gastrulation is only about 5 hours at 28.degree. C.; somites form between 10-20 hours; and by 24 hours postfertilization, a recognizable animal with rudimentary eyes and brain has formed. Thus, the early development of this vertebrate takes only about as long as a phage plaque assay. Rossant et al.(1992, Genes Dev., 6: 1) describe mutational strategies for mutagenesis of zebrafish, including ENU mutagenesis.

DEPR:
Non-mammalian organisms, such as fish, nematodes, and insects, are particularly useful according to the invention in identifying mutations in genes which are suspected to play a role in early development of the organism, e.g., in embryonic development, such as pattern-forming genes, limb-forming genes, or organ-forming genes.

DEPR:
The invention therefore contemplates the use of any type of mutagenesis technique, including chemical mutagenesis, radiation mutagenesis, and to mutagenesis techniques which are based on molecular biology, such as introduction into an organism of a gene encoding a defective DNA repair enzyme, retroviral insertion mutagenesis and promoter- and gene-trapping mutagenesis, as described below.

DEPR:
A) Chemical Mutagenesis and Mutagens.

DEPR:
Chemical mutagens are classifiable by chemical properties, e.g., alkylating agents, cross-linking agents, etc. The following chemical mutagens are useful according to the invention. The following four mutagens are particularly useful for mutagenesis of male germ cells:

DEPR:
One particularly useful mutagen according to the invention is the chemical mutagen ethylnitrosourea (ENU). ENU may be used to induce genomic mutations in any organism, including but not limited to lower organisms such as insects and worms, as well as higher organisms such as vertebrates, e.g., mammals, e.g., rodents such as mice and rats, hamsters, primates, and zebra fish, cows, sheep, pigs, and dogs. Mutagenesis and DNA mutation screen also may be applied to other organisms which are used as model systems for human disease. Rats are a good candidate for practical reasons, i.e., since mouse-based animal facilities are able to breed and maintain rats. The inventive methods are easily applicable to the rat and provides a method for producing and identifying mutations in specific rat genes.

DEPR:
Experiments in which mutagenesis was induced by the chemical mutagen ENU have used in excess of 500,000 mice. The genes involved were assayed indirectly by observation of phenotypic changes in the mice. ENU is believed to produce mutations at random throughout the genome, and the frequency of mutations, determined for numerous genes, is in the range of 0.5-1.5 mutant mice per 1000 mice, irrespective of the gene screened. In the past, the presence of mutations could only be inferred on the basis of a phenotype in the mutated mice. Most of these mutations do not produce an obvious phenotypic change in the heterozygous state and required additional breeding to make the mutations homozygous (F2 and F3 generations) to observe the effect of the mutation. Mutagenesis and the preparation of heteroduplex-enriched nucleic acid samples according to the invention does not require a previously-determined mutant phenotype, as the F1 generation mouse DNA is analyzed directly for the presence of a mutation in the gene of interest. In 1000 mice, 0.5-1.5 mutations in any gene may be detected. By screening 10,000 mice, it is possible to identify 5-15 mice, each carrying heterozygous mutations in a target gene. Any number of genes can be screened in these same 10,000 mice. Assuming 100,000 genes in a mammalian genome, then each mutagenized mouse is carrying mutations in one copy of approximately 100 different genes. The additional mutant genes in each mouse are easily removed by breeding. ENU mutagenesis of mice is performed as described in Example 1.

DEPR:
UV light-induced mutations are largely single nucleotide alterations. However, because UV light does not penetrate an animal, it is used for inducing mutations in cells in culture, including embryonic stem (ES) cells, or on exposed tissues of an animal, e.g., eyes, skin.

DEPR:
In addition to chemical or radiation induced mutations, mutations may be induced in an animal using insertional mutagenesis techniques, as follows.

DEPR:
McWhir et al. (1993, Nature Genetics, 5: 217) describe a mouse containing a defective DNA repair enzyme resulting from a mutation in the DNA repair gene ERCC-1. In nucleotide excision repair, damaged bases are removed with adjacent residues as an oligonucleotide and the resulting gap is then patched by repair synthesis. ERCC-1 is required for the incision step necessary to remove damaged DNA. Mice were generated containing the defective gene by targeting the excision repair cross complementing gene ERCC-1 in the embryonic stem cell line, HM-1. Homozygous ERCC-1 mutants died before weaning; however, heterozygous ERCC-1 mutants survived and were available for mating. It is contemplated according to the invention that a mammalian organism heterozygous for a mutant gene encoding a DNA repair enzyme may be used to screen for a mutation in a gene of interest.

DEPL:
Other chemical mutagens which are useful are as follows:

DEPU:
B. Ehling, U. H. & Neuhauser-Klaus, A., 1984, in Problems of Threshold in Chemical Mutagenesis, eds. Tazima, Y., Kondo, & Kuroda, Y. (Environ, Mutagen. Soc. Jpn., Mishima, Japan), pp. 15-25.

DEPV:
procarbazine hydrochloride

DETL:
TABLE I

Specific-locus mutation rates induced by chemicals that are mutagenic in post-cell stages of spermatogenesis Period of Induced mutation rate1 maximum Exposure2 per locus Lethal3/tested Chemical Ref. effect. days4 mg/kg mol .times. 10.sup.-5 .times. 10.sup.-5 5 per mol mutations

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Cp A 1-14 120 46.0 24.3 0.5 3/5 McMs 5-12 10/14 EtMs 5-12 0/1.1 Et.sub.2 SO.sub.4 C 5-12 4/4.1 AA 8-14 I 1/2.1 TEM 8-21D 7/8 346.2 ChI 15-21 1/438.7 Prc 8 1/4.1 ENU 32-38 0/5.2 MNU 36-42 0/17

Cp, cyclophosphamide; MeMS, methyl methanesulforate; EtMs, ethyl methanesulforate; Et.sub.2 SO.sub.4, diethyl sulfate; AA, acrylamide monomer; TEM, triethylene melamine; ChI, chlorambucil; Prc, phocarbazine hydrochloride; ENU, Nethyl-N-nitrosourea; MNU, Nmethyl Nnitrosourea. 1 Expressed per kg of body weight. When results for more than one exposur level of a chemical were available, we list the one that the investigator(s) found most suitable for generating mutationrate

data. 2Experimental minus historical control, 43/801, 406, for period of maximu response. 3Lethals excluded. For chlorambeuil, the number includes mutations for which there is genetic, cytogenetic, and/or molecular evidence for deletion. 4Postexposure. 5Number of mutations in sample is shown in parentheses. 8Experiment did not involve sequential matings.